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Immunoseparation of Prion protein enriched domains from other detergent-resistant membrane fractions, isolated from neuronal cells

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Abstract The possibility of coexistence of different subtypes of membrane lipid rafts has been investigated in cerebellar granule cells, by submitting detergent-resistant membrane fractions to immunoprecipitation. Among the proteins and lipids present in detergent-resistant fractions, almost all Prion protein, GAP43 and PKC were present in the immunoprecipitate obtained with anti-GAP43 or anti-Prion protein antibody at 4°C, together with a small fraction of cholesterol and sphingolipids, suggesting that they belong to a distinct subset of membranes. On the contrary, all Fyn and almost all MARCKS remained in the supernatant. Fluorescence microscopy experiments showed that Fyn and Prion protein were mostly not colocalized within a single neuron. Our results suggest that granule cells membranes contains different subtypes of detergent-resistant fractions, possibly deriving from different lipid rafts.

Key words: Neuron; Lipid raft; Prion protein; PKC; GAP43

1. Introduction

The concept that cell membranes are heterogeneous structures containing lipid microdomains, having a composition distinct from that of the bulk membrane, is generally accepted. A particular type of domains are lipid rafts, sphingolipid- and cholesterol-enriched structures involved in the modulation of fundamental events at the level of cell membranes [1]. The experimental clues of the existence of lipid rafts are considered to be the detergent-resistant membrane fractions (DRM), which can be separated at cold, and on the basis of their lower density by sucrose gradient centrifugation, and enriched in specific, often lipid-modified proteins [2].

The possibility of coexistence of different membrane domains, performing different biological functions within the same cell, has been already ascertained. Most of the information available on this topic is concerning the coexistence of *caveolae* besides *non-caveolar* domains [3,4]. For instance, such a proof has been obtained in endothelial cells using immunofluorescence confocal microscopy and immunoprecipitation techniques [3,5], in MDCK cells and human fibroblasts using electron microscopy [4], and in human intestinal epithelial cells [6] by selective immunoprecipitation with anti-glycosphingolipid antibodies.

An intriguing possibility that has been only partially investigated is whether or not different *non-caveolar* lipid rafts may coexist within the same cell. Some clues support this possibility: for instance, in model membranes distinct sphingolipid-enriched domains are detectable [7]; rabbit brain microsomal membranes contain different coexisting pools composed of ganglioside molecular species carrying different ceramide compositions [8]. We decided to utilize rat cerebellar granule cells (CGCs) differentiated in culture to investigate the coexistence of different non-caveolar membrane lipid rafts. In fact, in these cells, the presence of DRM has been repeatedly ascertained [9–12] while, concerning caveolae, their presence in CGCs, as in general in differentiated neurons, seems to be excluded [12–15]. In this work we show that at least two subtypes of DRM, having different protein and lipid compositions, are coexisting in CGC membranes.

2. Materials and methods

2.1. Chemicals

The reagents used (analytical grade) and HPTLC plates (Kieselgel 60) were purchased from Merck GmbH (Darmstadt, Germany). Modified Eagle's basal medium, fetal calf serum, trypsin, CAPS (3-[cyclohexylamino]1-propanesulfonic acid), 2-[*N*-morpholino]ethansulfonic acid (MES), and antibody against GAP43 were from Sigma Chem. Co. (Milano, Italy).

Primary antibodies against Fyn (sc-434), against MARCKS (sc-6455) and against Prion protein (PrP^C) (sc-7693) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the materials for the electrophoresis were from BioRad (Milano, Italy).

Secondary antibodies for immunofluorescence microscopy were Texas-Red donkey anti-goat and FITC donkey anti-mouse (Jackson Immunoresearch Lab, PA, USA). Secondary antibodies for enhanced chemiluminescence (ECL) detection were mouse and/or goat-HRP conjugates (Pierce).

2.2. Cell cultures

Granule cells, obtained from the cerebella of 8-day-old Sprague–Dawley rats (Charles River, Milan, Italy), were prepared as described [16,17]. Proliferation of glial cells was prevented by adding cytosine arabinofuranoside (final concentration, 10 μM) and checked by microscopic examination. Cell morphology was followed by microscopic examination and cell viability was monitored with fluorescein diacetate and propidium iodide [17]. The experiments were performed with cells cultured for 8 days in vitro (DIV). The protein content was determined with the micro BCA assay from Sigma Chem. Co. (Milano, Italy).

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Abbreviations: CGCs, cerebellar granule cells; DRM, detergent-resistant membrane fraction; MES, 2-[*N*-morpholino]ethansulfonic acid; PMSF, phenylmethylsulfonyl fluoride

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2.3. Preparation and characterization of DRM

Cells at 8 DIV were cultured in 100 mm dishes, washed two times, harvested in Locke's solution and submitted to centrifugation. In order to maintain a constant protein/detergent ratio, a cell pellet corresponding to 2.5 mg cellular proteins was incubated in 2 ml of 1% Triton X-100 in 25 mM MES buffer, pH 6.5, containing 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 75 units/ml aprotinin (MBS buffer), for 30 min on ice. The cell lysate was submitted to discontinuous sucrose density gradient centrifugation, as described [11,12]. Briefly, the cell lysate (2 ml) was diluted with an equal volume of 80% (w/v) sucrose in MBS lacking Triton X-100 and placed at the bottom of a discontinuous (30–5%, 4 ml each) sucrose concentration gradient in MBS without Triton X-100. After centrifugation at 250 000 × *g* for 18 h at 4°C, 1 ml fractions were collected and submitted to further analysis. From now on, to fraction #5 from the top they will be referred as DRM (detergent-resistant membrane fraction), to fractions from #9 to 12 as HDF (high density membrane fraction).

2.4. Radiolabeling of cell sphingolipids

Cells were incubated in the presence of 10^{-9} M [3-^{3}H]sphingosine (specific activity, 15 Ci/mmol, PerkinElmer Life Science), 5 ml/dish, in cell-conditioned medium for 2 h (pulse) followed by 48 h chase, according to Prinetti et al. [9]. DRM from [3-^{3}H]sphingosine-fed cells were prepared following the Triton X-100 method above described.

2.5. Immunoprecipitation experiments

All the following protocols were performed at 4°C. Aliquots of DRM (900 μl) obtained from different treatments were diluted 10-fold in immunoprecipitation buffer (IB) containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 75 mU/ml Leupeptin, 1% Triton X-100, precleared by incubation with 50 μl of protein G-coupled magnetic beads (Dynabeads Protein G) and placed in a rotary mixer for 2 h. To the supernatant, after separation of beads, was added 10 μg/ml anti GAP43, or 10 μg/ml anti-PrP^c, or 10 μg/ml normal mouse IgG (as negative control) and the mixtures stirred overnight at 4°C. Immunoprecipitates (IP) were recovered using 50 μl protein G-coupled magnetic beads for 4 h at 4°C, washed three times with IB buffer, recovered and suspended in Laemmli buffer. Fifty μl of the total supernatants (9 ml), remaining after immunoprecipitation, was dialyzed, lyophilized and suspended in Laemmli buffer.

In some comparative experiments, aliquots of the DRM were diluted in the above IB buffer and incubated at 37°C for 30 min to allow the desegregations of the sphingolipids-enriched domain [2,18] before performing the immunoprecipitation as described above.

Proteins in DRM, IP and in corresponding supernatants were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20, blots were incubated in the presence of antibody (anti-PKC 1:200, anti-Fyn 1:250, anti-PrP^c 1:500, anti-GAP43 1:500, anti-MARCKS 1:500), followed by reaction with secondary HRP-conjugates and ECL detection (Pierce Supersignal). The ECL film was submitted to densitometric scan. The digitized values of the spot corresponding to a given protein were utilized to express its partitioning between DRM and HDF as follows:

$$\text{Partitioning} = \frac{\text{digitized value in DRM} \times 100}{\text{average digitized values in HDF} + \text{digitized value in DRM}}$$

2.6. Lipid analysis

Lipids were extracted from IP and from the corresponding supernatants lyophilized, with chloroform/methanol 2:1 (v/v). Total extracts obtained from IP and 1/100 of the extract of the corresponding supernatants were analyzed by HPTLC. In the case of sphingolipid analysis, the solvent system was chloroform/methanol/0.2% aqueous $CaCl_2$ (55/45/10, v/v/v), followed by radioactivity imaging (Beta-Imager 2000 Z Instrument; Biospace, Paris, France). For cholesterol analysis, the extracted lipid samples were separated by HPTLC (solvent system hexane/diethylether/acetic acid 20/35/1, v/v/v) and then sprayed with anisaldehyde reagent. After heating the plate at 180°C for 5 min, the HPTLC plates were submitted to densitometric scanning. Quantification was made on the basis of known amounts of standard lipids loaded on the same plate.

2.7. Immunofluorescence microscopy

Cells grown on cover glasses were fixed for 10 min with methanol at −20°C, treated briefly with 0.1 M glycine in PBS (pH 7.4) followed by 0.3% Triton X-100, 15% filtered goat serum, 0.45 M NaCl, and 10 mM phosphate buffer, pH 7.4. Cover glasses were then incubated overnight at 4°C with primary antibodies (1:4), washed, and incubated for 1 h at room temperature with the appropriate conjugates secondary antibodies (1:100). Fyn was immunolocalized with the FITC-conjugated antibody. PrP^c was localized using the Texas-Red-conjugated antibody. Following a final washing, cover glasses were mounted with glycerol. Confocal microscopy was carried out on a Radiance 2100 microscope (Biorad Laboratories, Hercules, CA, USA) equipped with a krypton/argon laser; noise reduction was achieved by Kalman filtering during acquisition.

3. Results

Rat CGCs were submitted to the isolation of DRM. The evaluation of partitioning between DRM and HDF of Fyn, PrP^C, GAP43, PKC and MARCKS, calculated as described above, gave figures of 100, 100, 80, 30 and 15, respectively (Fig. 1), indicating that almost all cellular Fyn and PrP^C are concentrated in DRM, while only a smaller but significant portion of the other proteins is present in this fraction. It is worth noting that in the case of GAP43 and MARCKS, and differently from PKC, a significant amount of these proteins is also present in fractions having an intermediate density between DRM and HDF. It will be interesting to deepen the origin of this behavior.

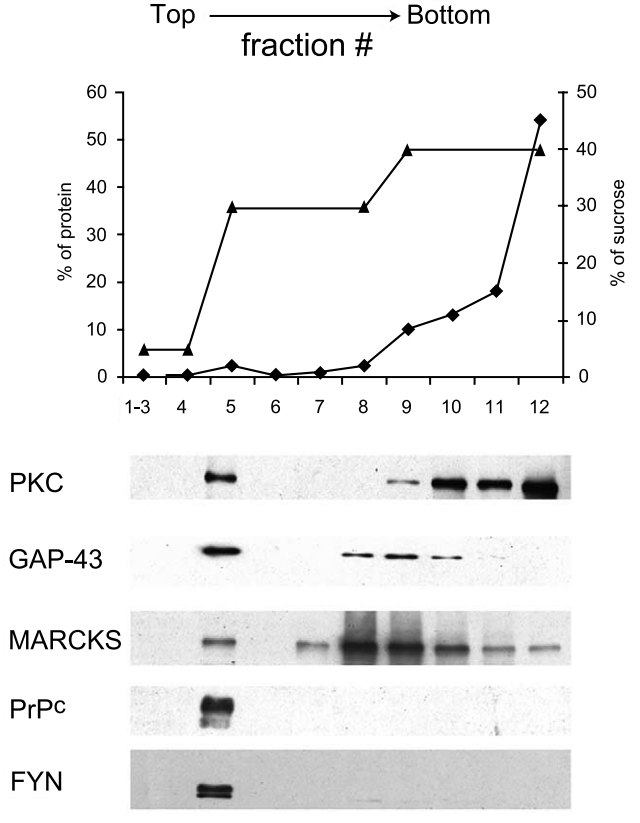

Fig. 1. Immunoblot analysis of PKC, GAP43, MARCKS, PrP^c and Fyn of sucrose gradient fractions, obtained from CGCs after treatment with 1% Triton X-100 at 4°C, and submitted to 10% SDS–PAGE. Except for fractions 1–3, which were pooled, for each fraction 5 μg protein/lane was loaded. The upper panel shows the protein distribution (diamonds) (% in each fraction) and the sucrose step gradient utilized (triangles).

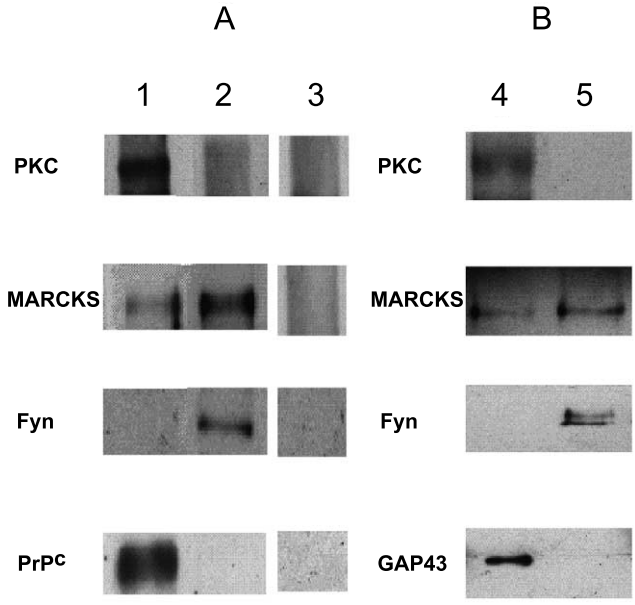

Fig. 2. Immunoblot analysis of PKC, GAP43, MARCKS, PrP^c and Fyn in IP obtained from detergent-resistant membranes using anti-GAP43 (panel A) or anti-PrP^c (panel B) antibodies at 4°C, and submitted to 10% SDS–PAGE. A: Lane 1, proteins associated with the IP; lane 2, proteins remaining in the corresponding supernatant; lane 3, proteins associated with G-coupled magnetic beads used to preclear the sample. B: Lane 4, proteins associated with the IP; lane 5, proteins remaining in the corresponding supernatant.

Successively, we analyzed the composition of IP obtained from DRM using antibodies against GAP43 (Fig. 2A). The IP proteins were submitted to electrophoresis and immunoblotting with anti-PrP^c, PKC, Fyn and MARCKS antibodies. The results show that all PrP^C and all PKC present in DRM were detected in the IP (lane 1). On the contrary, almost all Fyn and the major part of MARCKS present in DRM were recovered in the supernatant (lane 2). GAP43 was not detected in this supernatant (data not shown), indicating that all of this protein was in the IP. Control of protein G-coupled magnetic beads used to preclear (lane 3) indicated that immunoprecipitation was specific.

To confirm these results, we used antibody against PrP^C for immunoprecipitation of DRM (Fig. 2B). The results show that the IP obtained with anti-PrP^C (lane 4) contained all GAP43 and PKC present in DRM. On the contrary, almost all Fyn and the major part of MARCKS (about 95%, as calculated after correction for the amount of protein loaded on the electrophoresis) remained in the supernatant (lane 5). In this latter supernatant PrP^C was not detected (data not shown), indicating that all of this protein was in the IP.

To confirm that the information obtained by immunoprecipitation with anti-GAP43 or anti-PrP^C was due to the specific interaction of the antibody with the protein present in the domain, DRM was immunoprecipitated at 4°C with normal mouse IgG (Fig. 3, lanes 1 and 2) or with goat IgG (data not shown). Under these conditions none of the above said proteins was detected in the IP (lane 1), but remained in the supernatant (lane 2).

Additional experiments were performed under conditions that do not preserve the membrane resistance to detergents: DRM was warmed at 37°C, 20 min, before performing the immunoprecipitation using anti-GAP43. Fig. 3 (lanes 3 and 4) shows that under these conditions, PKC, MARCKS, Fyn, and PrP^C were not detected in the IP.

Table 1
Sphingolipid composition of detergent-resistant membranes (DRM), of immunoprecipitates (IP) and corresponding supernatants (S_{IP}) obtained from DRM with anti-GAP43 antibody

	DRM	IP	S_{IP}
SM	24.7	16.1	20.3
GM1	10.8	9.7	10.6
GD3	9.8	9.3	12.3
GD1a	14.8	15.4	16.8
GD1b	18	18.9	18.1
GT1b	22.1	30.4	22.2

Radioactive lipids were extracted and separated by HPTLC in the solvent system chloroform/methanol/0.2% aqueous $CaCl_2$ (55/45/10, v/v/v). Radioactive lipids were detected by digital autoradiography. Data are expressed as percent of radioactivity carried by each lipid over the total radioactivity present in each fraction.

As concerning the lipid composition of IP, the cholesterol content was analyzed by HPTLC of total lipid extracts from the IP. After comparison with standard cholesterol loaded on the plate and after correction for the amount of extract loaded, it was calculated that cholesterol present in IP was about 2.5% of the total DRM cholesterol.

To assess the sphingolipid content, total lipid extracts of the IP obtained from cells previously incubated in the presence of [3-^{3}H]sphingosine were analyzed by TLC. In Table 1 is reported the pattern of radioactive lipids present in DRM, in the IP and in the corresponding supernatant. The radioactive lipids pattern of IP shows small differences with respect to DRM, with a decrease of the proportion of sphingomyelin and an increase of GT1b ganglioside. Taking into account the total amount of radioactivity in DRM (about 200 000 dpm), in the IP (about 13 000 dpm) and in the corresponding

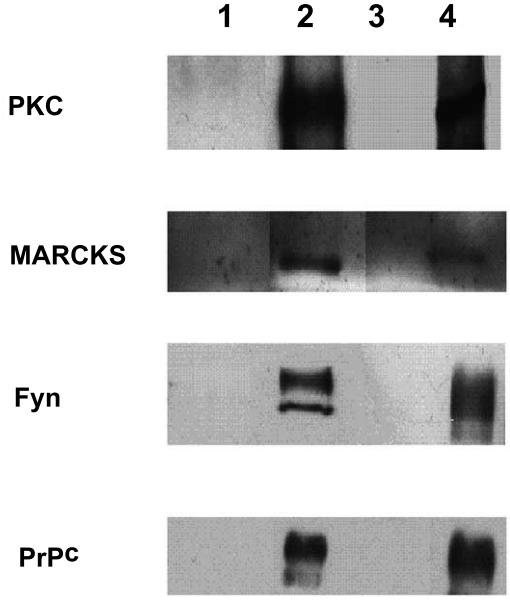

Fig. 3. Immunoblot analysis of PKC, MARCKS, PrP^c and Fyn of IP obtained from detergent-resistant membranes under different experimental conditions. Proteins associated with the IP (lane 1) and in the corresponding supernatant (lane 2), using normal mouse IgG at 4°C. Proteins associated with the IP (lane 3) and in the corresponding supernatant (lane 4) using anti-GAP43 at 37°C.

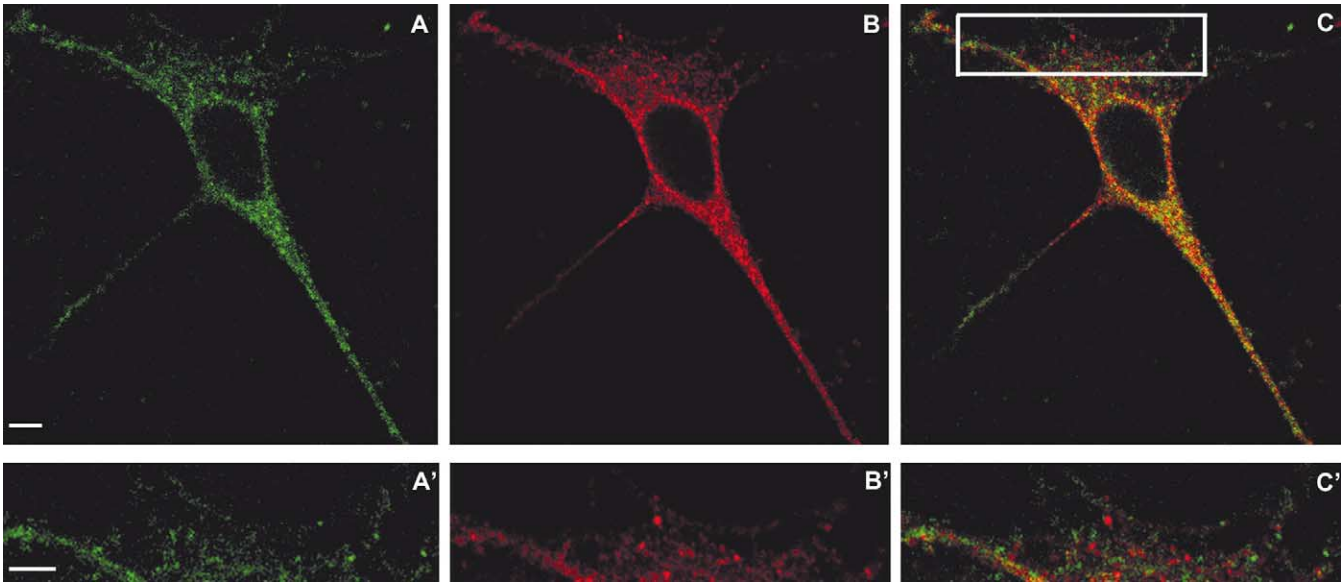

Fig. 4. Immunofluorescence microscopy analysis. The cells grown on glass coverslips, after methanol fixation, were double stained with the FITC-conjugated antibody (green, panel A) in the case of Fyn and with the Texas-Red-conjugated (red, panel B) antibody in the case of PrP^c. Merged confocal sections (panel C) showed only a partial colocalization of Fyn with PrP^c. Bar, 5 μm. Enlargement of a cell zone (delimited by a white line) shows that the two proteins are mostly not colocalized (panel C′). Bar, 5 μm.

supernatant (about 173 000 dpm), it was calculated that the amount of sphingolipids present in the '*Prion domain*' is about 6.5% of the total present in DRM. Control experiments carried out at 4°C with normal mouse IgG showed that under these conditions all the radioactivity was not in the IP, but in the corresponding supernatant (data not shown).

Finally, immunofluorescence confocal microscopy experiments were carried out with anti-Fyn and anti-PrP^C antibodies (Fig. 4). The observation at low magnification of a single cell showed a scattered distribution of both proteins at any cell level, nucleus excluded (Fig. 4A,B). Merged confocal sections (Fig. 4C) showed that Fyn and PrP^c are only partially colocalized. Observation at a larger magnification (Fig. 4A′–C′) allowed to establish that the two proteins are mostly not colocalized.

4. Discussion

The aim of the present study was to assess the presence in CGCs of different subtypes of lipid rafts having different protein and/or lipid composition. We chose this cellular system since caveolin has not been detected in CGC [12] and the protein and lipid composition of non-caveolar DRM isolated from these cells has been previously characterized [9–12,18].

The present investigation suggests that in DRM of CGC, *at least* two types of detergent-resistant membranes are present. The experimental support of this evidence is given by experiments carried out on DRM with antibodies against PrP^c and GAP43, showing coimmunoprecipitation of PrP^c and GAP43, and exclusion of Fyn and MARCKS from the IP.

Immunofluorescence confocal microscopy observations at a large magnification of zones where the cell is presumably thinner and where it is easier to discriminate among different membranes established that Fyn with PrP^c are present within the same cell and are mostly not colocalized. Parallel experiments carried out under conditions that do not preserve the integrity of domains, or using non-specific antibodies, indicated that the IP were specific. The compositional analysis showed all PrP^c, GAP43 and PKC contained in DRM were present in the IP, suggesting that these proteins belong to a distinct subset of DRM. Since DRM are considered to be the experimental clues of the existence of lipid rafts [2], these results suggest the presence in CGC of lipid rafts containing the above-mentioned proteins, and in particular almost all cellular PrP^C (*Prion domains*).

Concerning the functional significance of the colocalization of PrP^c and PKC in the Prion domain, it could be explained for two reason: first, PrP^c is a substrate of PKC (in vitro) and the phosphorylation/dephosphorylation cycle might modulate PrP^c biological activity [19]. Second, it was demonstrated that the physiological cleavage occurring between the 110/111–112 amino acid residues of PrP^c, is upregulated by effectors of the PKC pathway [20]. Also the GAP43 enrichment within the Prion domain could have a functional significance, since GAP43 in CGCs is a preferential substrate of PKC, and the amount of phosphorylated GAP43 promotes and regulates cell-surface dynamics, phagocytosis, cell attachment and regulated morphogenic processes such as neurite outgrowth [21].

Concerning the relative contribution of the Prion domain to DRM, it can be speculated that this domain represents only a minor portion of DRM, since the IP contained only a small portion of DRM cholesterol and sphingolipids, with a significant difference in the sphingolipid pattern. However, these issues deserve further investigations.

Regarding other proteins that are present in DRM, almost all Fyn (which represent virtually all the cellular proteins) and 95% of MARCKS were not in the Prion domain. This result finds a confirmation in a previous paper [21], suggesting that Scr tyrosine kinase are not associated to the GAP43-containing domain, but we have no further information. In line of principle, these proteins could be part of one or more other subsets of DRM. It can be reminded that Kasahara et al. [22] and Prinetti et al. [18] demonstrated that GD3, cholesterol, glycosphingolipids and different Src-proteins (Lyn, Fyn and c-

Scr) are coimmunoprecipitated from CGC by anti-GD3 antibody, indicating that these molecules probably share the same lipid rafts.

In conclusion, our results suggest that different subtypes of DRM, presumably deriving from different lipid rafts, can be isolated from CGCs.

In particular, it is possible that the '*Prion domain*' contains a specific set of signaling components and this restriction would limit access of the components of other signaling pathway (i.e. Fyn) and prevent non-specific signaling. The use of different antibodies against protein or lipid membrane domains fraction, together with the development of very sensitive analytical procedures, should allow us to obtained new pieces of information about the properties of different membrane lipid domains.

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